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Abstract

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Keywords

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Extracellular Chaperones and Amyloids

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ABSTRACT

The pathology of more than 40 human degenerative diseases is associated with fibrillar proteinaceous deposits called amyloid. Collectively referred to as protein deposition diseases, many of these affect the brain and the central nervous system. In many cases the amyloid deposits are extracellular and are found associated with newly identified abundant extracellular chaperones (ECs). Evidence is discussed which suggests an important regulatory role for ECs in amyloid formation and disposal in vivo. This is emerging as an exciting field. A model is presented in which it is proposed that, under normal conditions, ECs stabilize extracellular misfolded proteins by binding to them, and then guide them to specific receptors for uptake and subsequent degradation. In this scenario, EC receptors are a critical part of a quality control system which protects the brain against dangerously hydrophobic proteins/peptides. However, it also appears possible that in the presence of a high molar excess of misfolded protein, such as might occur during disease, the limited amounts of ECs available may actually exacebate pathology. Further advances in understanding of the mechanisms that control extracellular protein folding are likely to identify new strategies for effective disease therapies.

Keywords: Extracellular chaperones, receptor-mediated endocytosis, amyloid, protein quality control, aggregate toxicity, fibril formation

1.0 INTRODUCTION

The term "molecular chaperone" was first developed in the late 1970's when referring to the ability of nucleoplasmin to inhibit inappropriate interactions between histones and DNA (Laskey *et al.,* 1978). The meaning of this term is continuing to evolve but two key properties of molecular chaperones are (i) selective binding to non-native protein conformations to form stable complexes, and (ii) inhibition of the irreversible aggregation of non-native protein conformations (Fink 1999). Many molecular chaperones maintain other proteins in "folding-competent" conformations, which are returned to the native conformation by the involvement of other refolding chaperones. Previous studies have overwhelmingly focussed on intracellular molecular chaperones and their roles in protein folding within the cell. The discoveries that a range of serious human diseases are related to protein aggregation phenomena, and that molecular chaperones affect these processes, has led to a recent explosion of research in this area. The pathology of more than 40 human degenerative diseases is associated with the deposition of fibrillar proteinaceous aggregates called amyloid. Collectively referred to as *protein deposition diseases*, these include various sporadic (e.g. Alzheimer's (AD) and Parkinson's (PD) diseases), familial, and transmissible degenerative disorders (e.g. spongiform encephalopathies such as Creutzfeldt-Jakob disease (CJD)), many of which affect, amongst other tissues and organs, the brain and the central nervous system (CNS). In many cases, molecular chaperones have been found physically associated with amyloid deposits, although the reason for this association remains to be established.

It is notable that many neurodegenerative disorders are associated with *extracellular* amyloid deposits. It has only recently become apparent that extracellular counterparts to the intracellular molecular chaperones exist. These proteins share functional characteristics with the small heat shock proteins in that they are able to efficiently stabilize misfolded proteins to prevent them aggregating but are not capable of independently refolding such proteins. Strikingly, in all cases tested, at least one or more of these "extracellular chaperones" (ECs) are associated with amyloid deposits found outside cells (Table 1). The role of these abundant ECs in the formation of amyloid deposits *in vivo* is emerging as an exciting field. In this chapter, we first briefly discuss what is understood about the molecular basis of amyloid formation before examining the available evidence that ECs influence this process and discussing the potential roles of ECs in amyloid formation and disposal *in vivo*.

2.0 AMYLOIDS AND THE BRAIN

2.1 Amyloid formation

The hallmark of a wide range of debilitating and incurable human pathologies is the abnormal presence of extracellular deposits in a variety of organs and tissues, including the brain and CNS (e.g. AD, CJD), peripheral organs such as the heart, liver, and spleen (e.g. systemic amyloidoses), and skeletal tissues and joints (e.g. haemodialysis-related amyloidosis). The extent of *in vivo* deposition can vary significantly depending on the disease state as well as the location of the deposits. For instance, deposits associated with neurodegenerative diseases of the brain typically weigh as little as a few grams whilst systemic deposits can accumulate to quantities of as much as several kilograms (Pepys 1995).

Amyloid fibrils arise when a specific protein or protein fragment converts from an otherwise soluble form into insoluble filamentous aggregates. Key fibril-forming

proteins associated with amyloid deposits and diseases have included full-length globular proteins, bioactive peptides (e.g. calcitonin), and fragments of whole proteins produced by specific cellular processing (e.g. the processing of amyloid precursor protein (APP) to yield Aβ) or by natural degradation. In recent years, it has become apparent that the self-assembly of proteins into amyloid fibrils is not restricted to the relatively small number of proteins associated with the protein deposition diseases. An increasing body of evidence points to this phenomenon as being a generic property of polypeptide chains, suggesting that most, if not all, proteins have the potential to form amyloid fibrils if exposed to appropriate conditions (Dobson 1999). What triggers the *in vivo* conversion of a protein from its native state into amyloid fibrils is not known, however, several important determinants of the fibrillation of proteins have been identified, including hydrophobicity and net charge (Chiti and Dobson 2006). At present, a detailed understanding of the mechanism for the transition from soluble precursors to mature amyloid fibrils is lacking. Despite this, it seems that the aggregation behaviours of various amyloid-forming peptides and proteins are strikingly similar. Often described as a hierarchical process involving multiple stages of assembly, the kinetics of amyloid formation (as measured by ThT fluorescence or light scattering) is generally characterized by an initial 'lag' or nucleation phase, followed by a rapid exponential 'growth' or polymerization phase (Jarrett and Lansbury 1993), and lastly, by a plateau phase in which no further polymerization occurs (Fig. (1)).

The lag (or nucleation) phase is defined as the time required for the formation of the soluble (prefibrillar) oligomers or nuclei. This phase begins with the destabilization and partial unfolding of the native protein, leading to the formation of an ensemble of intermediately folded species. *In vitro* studies show that structural perturbation leading to protein unfolding can be achieved by exposing the native protein to one or more chaotropic conditions, including elevated temperature, low pH, oxidative stress, molecular crowding, and protease-mediated degradation (Kelly 1998). *In vivo*, the fate of the proteins within the ensemble of partially unfolded intermediates depends on the environmental conditions present at the time of their existence. Some proteins may be re-folded, whilst others may be degraded. A subset of proteins within this ensemble will undergo non-ordered aggregation to form nuclei. When viewed by transmission electron (TEM) or atomic-force (AFM) microscopy, these species may appear as small beads that are linked together to produce what are often described as amorphous aggregates or micelles (Yong *et al.,* 2002). The nucleation phase, which constitutes the rate-limiting step in amyloid formation, is relatively slow, due largely to unfavourable protein association equilibria rather than to intrinsically slow association rates (Jarrett and Lansbury 1993). After nucleation and immediately preceding the polymerization phase, the nuclei transform into an ensemble of various assemblies called protofibrils that exhibit increased levels of complexity and order. During the polymerization phase, these protofibrils rapidly grow via the addition of either monomers or oligomers that are formed after the initiation of the aggregation process, leading to the formation of well-ordered protofilaments. The lateral association of such protofilaments, accompanied by minor structural reorganization, is thought to give rise to the formation of mature amyloid fibrils (van Gestel and de Leeuw 2007).

Regardless of their individual protein composition, the molecular architecture of all amyloid fibrils shares some striking similarities. Despite a lack of information

detailing the structure of amyloid fibrils at the atomic level, techniques such as X-ray fibre diffraction, solid-state NMR, and cryoelectron microscopy have given insights into how proteins pack together to form such highly ordered structures. Typically, amyloid fibrils exist as long, unbranched, but often twisted structures that are 6-12 nanometres in diameter, bearing a characteristic 'cross-β' X-ray fibre diffraction pattern (Sunde and Blake 1997). Each fibril is usually comprised, within its core structure, of two to six 'protofilaments' that wind around each other to form supercoiled rope-like structures. Protofilaments are believed to be principally stabilized by intra- and intermolecular interactions (e.g. hydrogen bonds) and are composed of β-sheets whose strands lie perpendicular to the long axis of the fibril (Serpell 2000; Serpell *et al.,* 2000).

2.2 Amyloid pathology in the brain

Protein deposition diseases are typically characterized by protein aggregates in specific regions of the brain, neuronal death and brain function decline. In the case of AD, pathological symptoms include both large anatomical and microscopic changes, and are generally characterized by gross diffuse atrophy, primarily in the frontal, temporal and parietal regions of the cortex, and extensive neuronal loss. Microscopic features, commonly detected by histological staining procedures, include extracellular amyloid plaques and intracellular neurofibrillary tangles (Gorevic *et al.,* 1986). Pathological features of PD, on the other hand, include the accumulation of Lewy bodies within the cytoplasm of pigmented neurons of the cerebral cortex. Yet, for CJD, no obvious gross pathology, besides neuronal loss and gliosis, is ever observed due to the short course of the lethal disease. Despite advances in understanding of the pathological basis of the various amyloid-related neuropathies, the actual cause/s of disease remains undetermined and are the subject of intense investigation. Numerous theories have been advanced to account for the pathology of amyloid diseases. Many of these theories particularly focus on the toxic nature of amyloids and how this relates to neuronal loss in the brain (reviewed in (Stefani and Dobson 2003)).

One theory proposes that disease pathology might simply be attributed to the shear bulk of deposited material present in the affected organs and tissues, such as the brain. It suggests that the extensive accumulation of amyloid deposits causes physical disruption to the cellular architecture, subsequently leading to organ dysfunction. Another theory suggests that the formation of pores or channels in neuronal cells by oligomeric species formed from the self-association of partially unfolded proteins may be a critical determinant of amyloid-related neuropathy. Consistent with this idea, atomic force microscopy (AFM) has shown that Aβ, the primary constituent of senile plaques, can form pore-like structures *in vitro* (Chromy *et al.,* 2003; Lashuel *et al.,* 2003). In addition, ion flux data indicate that synthetic Aβ can form cation-selective channels across model membranes, leading to increased membrane permeability, disruption of calcium homeostasis and associated cellular degeneration (Arispe *et al.,* 1993). Similar pore forming activity has also been reported for amylin, the major component of type-II diabetes-associated islet β-amyloid (Mirzabekov *et al.,* 1996) and Cu/Zn superoxide dismutase (SOD1), which is associated with amyotrophic lateral sclerosis (ALS) (Chung *et al.,* 2003).

A more recent and widely accepted theory proposes that soluble, inherently toxic species are the primary cause of pathogenesis in disorders such as AD. Until recently, neuronal cell death was attributed to the presence of mature fibrils, the most common

form of proteinaceous aggregates present in pathological deposits. Yet, an increasing quantity of data suggests that the highly toxic species are in fact the pre-fibrillar structures (e.g. oligomers and protofibrils), rather than the mature fibrils into which they develop. Exemplifying this, small, soluble and diffusible Aβ oligomers (often refered to as amyloid β-derived diffusible ligands or ADDLs) have been shown to be highly toxic to cultured neuronal cells, even at nanomolar concentrations (Dahlgren *et al.,* 2002). The demonstration that these species are, in addition to being highly cytotoxic, readily diffusible throughout the brain may explain why neuronal loss is commonly observed at sites distant from those of the amyloid deposits. By proposing that the primary toxic species are the early aggregates, this theory also provides a plausible explanation for the lack of correlation between the extent of deposition of mature fibrils in the form of amyloid plaques in the diseased brain and the severity of clinical symptoms (Katzman *et al.,* 1988).

Protein aggregation is often accompanied by the production of reactive oxygen species (ROS), although the reason/s for this is not entirely understood. There is evidence to indicate that oxidative stress, arising from the ageing process or from the production of ROS, contributes to disease pathology. The observation that Aβ deposition in the brain is positively correlated with regions of oxidative stress and neurodegeneration supports this notion (Hensley *et al.,* 1995). Lending further support is the demonstration that antioxidants such as lipoic acid and resveratrol can protect cells against aggregate-mediated toxicity (Savaskan *et al.,* 2003; Jesudason *et al.,* 2005). Disease pathology is believed to arise because oxidative stress reduces the expression levels and activity of the multicatalytic proteosome (MCP), which is normally responsible for ubiquitin ATP-dependent degradation of aberrant protein structures. The reduction of available MCP leads to the accumulation of oxidized and damaged proteins at levels that are sufficient to induce neuronal cell death (Keller *et al.,* 2000).

2.3 Potential control mechanisms?

Inside cells the collective functions of chaperones, the ubiquitin-proteasome system and lysosome-mediated autophagy is usually sufficient to prevent accumulation of misfolded proteins. However, under certain conditions the capacity of this quality control system is exceeded and protein aggregates accumulate (Muchowski and Wacker 2005). Intracellular levels of molecular chaperones in the brain are thought to be lowered with increasing age (Hay *et al.,* 2004), which may contribute to the often late onset of protein deposition diseases. Overexpression of intracellular heat shock proteins (HSPs) in the nematode *Caenorhabditis elegans* promotes longevity, and in the same system a reduction in the level of heat shock factors (transcriptional activators) and thus HSPs shortens lifespan (Westerheide and Morimoto 2005). These results suggest that intracellular chaperones are vital in the continuous fight against misfolded protein aggregation and amyloid formation. As a corollary, it appears likely that in diseases involving extracellular protein deposition, the extracellular processes that normally operate to inhibit protein aggregation and to clear protein deposits may be impaired - they are certainly overwhelmed. This is supported by the observation that, apart from people carrying mutations that result in increased production of Aβ (e.g. presenilins or APP genes), the rate of formation of Aβ does not increase with age (Deane *et al.,* 2004) but its normally efficient removal from the cerebrospinal fluid (CSF) is slowed in aged humans (Zlokovic *et al.,* 2000).

Understanding of the mechanisms controlling protein folding in extracellular spaces, especially in the CNS, is in its infancy. However, there is evidence to suggest that there are such mechanisms. For example, although CSF is regularly released into the venous system through the arachnoid villi, the half lives of proteins in the CSF differ from protein to protein, and even differ between various proteolytic fragments of the same original protein (Savage *et al.,* 1998), suggesting a selective mechanism of removal. Recently it was proposed that there is an extracellular protein quality control system consisting of abundant secreted ECs that recognise and facilitate the disposal of non-native or dangerously hydrophobic proteins via receptor-mediated cell uptake (Yerbury *et al.,* 2005). These proteins may patrol the CNS for misfolded proteins and under normal conditions prevent their aggregation and mediate their clearance.

3.0 EXTRACELLULAR CHAPERONES

In the extracellular environment, protein concentrations may be high, as in human plasma (about 70 mg/ml), or lower such as in human CSF (120-640 μg/ml) (Williams and Marshall 2001). Exposure to stresses capable of inducing protein unfolding is common (e.g. sheer stress in extracellular fluids, hyperthermia, oxidative stress, transient pH fluctuations), and ATP is 1000 times less abundant than inside cells (Farias *et al.,* 2005). In the event of a large-scale presentation of extracellular non-native protein(s), such as might occur during amyloid disease, only ATP-independent, physically abundant extracellular chaperone(s) could reasonably be expected to provide an effective line of defense. Intracellular chaperones (e.g. Hsp70, Hsp90) are present in human plasma and CSF and are associated with cell surfaces (in particular cancer cells); they are thought to be released from dead or dying cells. Many potentially important extracellular roles have been proposed for these chaperones, such as cancer cell invasiveness (Eustace *et al.,* 2004), immune presentation (Becker *et al.,* 2002) and signaling (Whittall *et al.,* 2006). However, these "normally intracellular" chaperones are present extracellularly at very low (ng/ml) levels and require ATP to carry out protein refolding. Thus, the capacity of the low levels of Hsp70 and similar chaperones present extracellularly would be quickly exceeded. Therefore, it is far more likely that bulk processing of non-native proteins is dealt with by much more abundant proteins with chaperone properties, herafter referred to as extracellular chaperones (ECs), which have only recently been identified.

Four secreted glycoproteins, clusterin (Humphreys *et al.,* 1999), haptoglobin (Yerbury *et al.,* 2005), α2-macroglobulin (French *et al.,* 2007) and serum amyloid P component (SAP) (Coker *et al.,* 2000), have been shown to exhibit chaperone properties *in vitro*. These four proteins share some notable similarities. All four are secreted glycoproteins widely distributed in most physiological fluids, including plasma and CSF (Barrett 1981; Baltz *et al.,* 1982; Bowman and Kurosky 1982; Humphreys *et al.,* 1999). Moreover, all bind to a broad range of ligands (Barrett 1981; Capiau *et al.,* 1986; Katnik *et al.,* 1987; Aruga *et al.,* 1993; Katnik *et al.,* 1993; Zahedi 1996; Ashton *et al.,* 1997; Langlois *et al.,* 1997; Zahedi 1997; Kurdowska *et al.,* 2000; Wilson and Easterbrook-Smith 2000; Kimura *et al.,* 2001; Sen and Heegaard 2002) and have been found associated with clinical amyloid deposits *in vivo* (Table (1)) (Powers *et al.,* 1981; Baltz *et al.,* 1982; Van Gool *et al.,* 1993; McHattie and Edington 1999; Calero *et al.,* 2000). In addition all have been shown to mediate receptor-mediated endocytosis of ligands (see below). In terms of the chaperone action, clusterin is currently the best characterized.

3.1 Clusterin

Clusterin is a protein secreted from many different cell types that is found in human plasma, CSF and seminal fluid at concentrations of approximately 100, 2, and 1000 μg/ml (Fritz *et al.,* 1983; Murphy *et al.,* 1988; Choi-Miura *et al.,* 1992), respectively. It can also transit from the cell secretory system to the cytosol during certain stress conditions, although the reasons why this occurs remain to be established (Nizard *et al.,* 2007). Clusterin mRNA is nearly ubiquitous in animal tissues, being found in locales as diverse as the rat prostate gland and quail neuroretinal cells. The expression of clusterin is increased in a wide variety of models of stress and disease, including withdrawal of growth factors and exposure to noxious agents (Jenne and Tschopp 1992). The clusterin promoter contains a highly conserved 14 bp element which is recognized by the transcriptional regulator heat shock factor 1 (HSF1) (Michel *et al.,* 1997). HSF1 activates expression of heat shock proteins (which protect cells from stresses) and clusterin (Michel *et al.,* 1997). Across different mammalian species, the amino acid sequence of the protein is maintained at the level of 70-80% (Jenne and Tschopp 1992). Clusterin is encoded by a single gene and the translated product is internally cleaved to produce its α and β subunits prior to secretion from the cell. Matrix-assisted laser desorption ionization mass spectrometry has identified two primary forms of human plasma clusterin at about 58 kDa and 63.5 kDa, which probably represent different glycoforms. The exclusively N-linked glycosylation is variable in nature and extent, ranging from 17-27% (by weight) (Kapron *et al.,* 1997). The function of the glycosylation is unknown, although it may be involved in recognition by cell surface receptors (Stewart *et al.,* 2007). Probably because of the substantive and variable glycosylation, when analysed by SDS PAGE, clusterin migrates as a broad band corresponding in position to a mass of 70-80 kDa. In aqueous solution at physiological pH, clusterin exists in a range of oligomeric forms; mildly acidic pH favours partial dissociation of oligomers into individual α-β heterodimers (Hochgrebe *et al.,* 2000). Current insights into clusterin structure are largely reliant upon predictions based on sequence analyses, which suggest that the protein has significant contiguous regions of disordered (possibly molten globule) conformation that separate other regions of well-defined secondary structure, such as amphipathic α-helical regions and coiled-coil α-helices (Dunker *et al.,* 2001; Dunker *et al.,* 2002). On this basis, clusterin has been categorized as an intrinsically disordered protein (Dunker *et al.,* 2001; Dunker *et al.,* 2002). The high level of disorder, variable glycosylation and tendency to form oligomers have so far limited attempts to structurally characterise clusterin by approaches such as NMR, X-ray crystallography and mass spectrometry.

Recent studies have demonstrated that clusterin has chaperone activity with a potent ability to influence the amorphous and fibrillar aggregation of many different proteins. Clusterin potently inhibits stress-induced protein aggregation by ATP-independent binding to exposed regions of hydrophobicity on non-native proteins to form soluble, high molecular weight complexes (Humphreys *et al.,* 1999; Poon *et al.,* 2000; Yerbury *et al.,* 2007). Immunoaffinity depletion of clusterin from human plasma renders proteins in this fluid more susceptible to aggregation and precipitation (Poon *et al.,* 2002). Clusterin lacks the ability to independently refold heat-stressed, non-native enzymes but, like the small heat shock proteins, is able to preserve heat-inactivated enzymes in a state competent for subsequent ATP-dependent refolding by Hsc70 (Poon *et al.,* 2000). However, because there is no currently known abundant

refolding-competent EC, the physiological significance of this remains uncertain. During amorphous aggregation of proteins, clusterin appears to interact with slowly aggregating species on the off-folding pathway.

By complexing with misfolded extracellular proteins, ECs like clusterin may mediate their cellular uptake and degradation (Yerbury *et al.,* 2005). Clusterin has long been known to interact with the cell surface receptor megalin (LRP2) and to complex with Aβ to mediate its uptake by megalin and subsequent degradation (Hammad *et al.,* 1997). It also interacts with other members of the low density lipoprotein (LDL) receptor family - it binds to chicken LR8 and an LDLR-related protein (Mahon *et al.,* 1999), and uptake of clusterin-leptin complexes by apoER2 and VLDLR has been proposed to facilitate leptin clearance (Bajari *et al.,* 2003). Furthermore, clusterin and LRP1/megalin have been implicated in the clearance of cellular debris by non-professional phagocytes (Bartl *et al.,* 2001).

3.2 Haptoglobin

Haptoglobin (Hp) is a secreted acidic glycoprotein (20% of its total mass is N-linked carbohydrate (Bowman and Kurosky 1982)) produced mainly in the liver and found in most body fluids of humans and other mammals. Normally, it is present in human plasma at 300-2000 μg/ml (Bowman and Kurosky 1982) and CSF at 0.5-2 μg/ml (Sobek and Adam 2003). However, the levels of Hp in human plasma are increased up to 8-fold during inflammation, various infections, trauma, tissue damage and in association with neoplasia, leading to it being designated as an "acute phase protein" (Bowman and Kurosky 1982; Dobryszycka 1997). Hp is encoded by a single gene; uniquely, in humans, there are two principal alleles (Hp1 and Hp2), which results in individual humans expressing one of three major Hp phenotypes (Hp 1-1, Hp 2-1, Hp 2-2). In all cases, Hp can be represented as a multimer of an αβ subunit. In its simplest form (Hp 1-1), Hp consists of a disulfide-linked $(\alpha 1)_2\beta_2$ structure (~100 kDa). However, in Hp 2-1 and Hp 2-2, an additional cysteine residue in the α2 chain allows the formation of a complex series of various sized disulfide-linked αβ polymers (~100 to ~500 kDa).

Hp binds with extremely high affinity to hemoglobin (Hb) ($K_D \sim 10^{-15}$ M; (Bowman and Kurosky 1982)). Formation of the Hp-Hb complex inhibits Hb-mediated generation of lipid peroxides and hydroxyl radical, which is thought to occur in areas of inflammation (Dobryszycka 1997). Although it was previously thought that Hp cleared the body of vascular Hb released from damaged red blood cells, recent work has shown that the ablation of Hp expression in mice had no significant effect on Hb clearance following experimentally induced severe hemolysis. Nevertheless, the Hp knock-out mice suffered substantially greater mortality under these conditions, which was attributed to greater oxidative stress (Lim *et al.,* 1998). Therefore, it appears likely that Hp exerts an important anti-inflammatory action *in vivo* by inhibiting oxidative damage mediated by free Hb (Lim 2001). A variety of other putative functions have also been ascribed to Hp. It has been implicated in immune regulation (Louagie *et al.,* 1993), shown to inhibit cathepsin B activity (Snellman and Sylven 1967) and to have pro-angiogenic effects (Cid *et al.,* 1993). Binding of Hp to human neutrophils has been reported to inhibit respiratory burst activity (Oh *et al.,* 1990). In addition, neutrophils have been shown to take up exogenous Hp and store it within cytoplasmic granules - they subsequently secrete it into the local extracellular environment in response to a variety of pro-inflammatory stimuli (e.g. yeast, TNFα,

or the chemotactic peptide fMLP (Wagner *et al.,* 1996; Berkova *et al.,* 1999)). Thus, the available evidence indicates that Hp is likely to play an important role in suppressing inflammatory responses.

Human Hp specifically inhibits the precipitation of a wide variety of proteins induced by a range of stresses (Pavlicek and Ettrich 1999; Yerbury *et al.,* 2005). All three human Hp phenotypes exert this chaperone action, although at equivalent mass concentrations, at least for one substrate protein tested, Hp1-1 was the most efficient. Like clusterin, Hp forms stable, soluble high molecular weight complexes with misfolded proteins. Also like clusterin, Hp lacks ATPase activity and has no independent ability to refold misfolded proteins. The possibility that Hp holds misfolded proteins in a state competent for refolding by other chaperones is currently untested. Immunoaffinity depletion of Hp from human serum significantly increased the amount of protein that precipitated in response to stresses (Yerbury *et al.,* 2005). Thus, Hp has the ability to protect many different proteins from stress-induced amorphous precipitation and its effects in whole human serum suggest that this activity is likely to be relevant *in vivo*. Currently, there are no published studies of the effects of Hp on amyloid formation, although Hp is found associated with amyloid deposits *in vivo* (Table (1)).

When complexed to Hb, Hp is known to bind to the CD163 cell surface receptor (Graversen *et al.,* 2002). Other receptors to which Hp alone binds are the CD11b/CD18 integrin (Mac-1/CR3), which also binds denatured proteins and the iC3b fragment of complement (Ross 2000), and the CD22 B lymphocyte receptor. Unidentified specific Hp binding sites have also been reported to occur on neutrophils (Oh *et al.,* 1990) and mast cells (El-Ghmati *et al.,* 2002). Thus, it appears feasible that Hp might interact with one or more of these receptors to mediate the clearance and degradation of misfolded extracellular proteins.

3.3 α_2-Macroglobulin (α_2M)

α_2-Macroglobulin (α_2M) is a major human blood glycoprotein, comprised of ~10% carbohydrate by mass. It is assembled from four identical 180 kDa subunits into a 720 kDa tetramer; the 180 kDa subunits are disulphide bonded to form dimers, which non-covalently interact to yield the final tetrameric quaternary structure (Jensen and Sottrup-Jensen 1986). α_2M is present in human plasma and CSF at 1500-2000 (Sottrup-Jensen 1989) and 1-3.6 μg/ml (Biringer *et al.,* 2006), respectively. It has a well known ability to inhibit a broad spectrum of proteases, which it accomplishes using a unique trapping method. When exposed to a protease, α_2M undergoes limited proteolysis at its bait region leading to a large conformational change, physically trapping the protease within a steric "cage" (Sottrup-Jensen 1989). The trapped protease forms a covalent linkage with α_2M by reacting with an intramolecular thiol ester bond to yield a conformationally altered form known as "activated" or "fast" α_2M (α_2M*), which exposes a receptor recognition site for low density lipoprotein receptor related protein (LRP) (Sottrup-Jensen 1989). By directly interacting with the thiol ester bond, small nucleophiles such as methylamine can also activate α_2M (Imber and Pizzo 1981).

Aside from its interactions with proteases, α_2M binds to Aβ peptide and β_2-microglobulin, which are associated with Alzheimer's disease (Narita *et al.,* 1997)

and dialysis related amyloidosis (Motomiya *et al.,* 2003), respectively, to cytokines and growth factors (Mettenburg *et al.,* 2002), and to a range of hydrophobic molecules including endotoxin, phenyl-Sepharose, and liposomes (Barrett 1981). The binding to hydrophobic molecules does not inhibit the trapping of proteases and is not known to be associated with any conformational changes (Barrett 1981). Previous work has indicated that α_2M-polypeptide complexes are immunogenic (Chu and Pizzo 1993; Binder *et al.,* 2001). α_2M bound peptides are internalised by LRP and fragments of the peptide are subsequently re-presented on the cell surface. This response is identical to the one elicited by peptides chaperoned by intracellular heat shock proteins (Srivastava 2002). A further hint that α_2M might have chaperone properties came from the observation that it inhibits the aggregation of Aβ and protects cells from Aβ toxicity (Du *et al.,* 1997). It was recently shown that α_2M has a promiscuous ATP-independent chaperone action similar to that of both clusterin and haptoglobin. It forms stable complexes with misfolded proteins to inhibit their stress-induced aggregation and precipitation but is unable to independently effect their refolding (French *et al.,* 2007). α_2M is the first known mammalian protein with both protease inhibitor and chaperone-like activities.

Selective removal of α_2M from whole human serum renders proteins in this fluid more susceptible to precipitation, even at 37 ^{o}C (French *et al.,* 2007), suggesting that the *in vitro* chaperone properties of α_2M are likely to be relevant *in vivo*. The effects of clusterin, haptoglobin and α_2M on plasma protein precipitation are additive (French *et al*., (2007) and unpublished data), suggesting that even though they are promiscuous in their interactions with different substrate proteins, the ECs may provide complementarity with respect to the endogenous extracellular proteins they protect. Interaction with a misfolded substrate protein does not activate α_2M and the resulting complex is not bound by LRP. However, if the complex subsequently interacts with and traps a protease, then the activated conformation is adopted and the α_2M/protease/misfolded protein complex is bound by LRP (French *et al.,* 2007). Thus, for example, interaction with abundant proteases at sites of inflammation may be one *in vivo* switch to trigger LRP-mediated uptake of α_2M/misfolded protein complexes. Although LRP is the only known receptor for α_2M, it remains possible that non-activated α_2M/misfolded protein complexes are taken up via other currently unknown cell surface receptors. Like the other ECs, it has been proposed that α_2M patrols extracellular spaces for misfolded proteins and facilitates their disposal via receptor mediated cellular uptake. This activity would contribute to important anti-inflammatory actions of α_2M *in vivo*.

3.4 Serum amyloid P component

SAP is a member of the pentraxin family of proteins which are characterised by five identical subunits noncovalently associated to form a disc-like structure. The SAP pentamer consists of five 25 kDa subunits each containing 204 amino acids and a single intra-chain disulfide bond, and constructed from multiple anti-parallel β-strands arranged in two sheets (Emsley *et al.,* 1994). Each human SAP subunit bears an invariant N-linked biantennary oligosaccharide which constitutes more than 8% of the mass of the molecule (Pepys *et al.,* 1994). It has been proposed that SAP circulates as a decamer with two pentameric discs noncovalently bound face to face (Wood *et al.,* 1988; Emsley *et al.,* 1994). Other reports claim that SAP exists as a single pentamer

in the body and that the decameric form is obtained only upon purification (Sorensen *et al.,* 1995; Aquilina and Robinson 2003). SAP is synthesised and catabolized in the liver, and is present in human plasma and CSF at concentrations of ~ 40 μg/mL (Hutchinson *et al.,* 1994) and 8.5 μg/ml (Hawkins *et al.,* 1994), respectively. Whereas SAP is an acute-phase protein in mice (Pepys *et al.,* 1979), in humans it's plasma concentration is not significantly elevated during acute inflammation (Pepys *et al.,* 1978).

Each subunit monomer has two Ca^{2+}-binding sites and shows Ca^{2+}-dependent binding to many different ligands, including certain oligosaccharides, glycosaminoglycans (Hamazaki 1987), fibronectin (de Beer *et al.,* 1981), C-reactive protein (Swanson *et al.,* 1992), aggregated IgG (Brown and Anderson 1993), C1q (Sorensen *et al.,* 1996), complement C4-binding protein (Sorensen *et al.,* 1996), DNA (Pepys and Butler 1987), chromatin (Breathnach *et al.,* 1989), histones (Hicks *et al.,* 1992), and phosphoethanolamine-containing compounds such as phosphatidylethanolamine (Emsley *et al.,* 1994). Interaction with these ligands localises SAP to elastic microfibrils (Breathnach *et al.,* 1981), glomerular and alveolar basement membrane, arterioles, bronchioles, sarcolemma of cardiac and smooth muscle (Dyck *et al.,* 1980), and all forms of amyloid (Pepys *et al.,* 1979).

Previous studies of the chaperone properties of SAP are insufficient in number and depth to allow unequivocal classification of SAP, together with clusterin, haptoglobin and α_2M as a genuine EC. However, one study showed that when added to a refolding buffer containing denatured lactate dehydrogenase (LDH), SAP markedly enhanced the yield of active LDH. The reaction was supra-stoichometric, as a ratio of SAP pentamer to LDH substrate of 10:1 was needed to recover 25% of enzyme reactivity (Coker *et al.,* 2000). This suggests that SAP is an inefficient refolding-competent chaperone. It is important to note that this *in vitro* activity was demonstrated in the absence of ATP and any "helper" chaperones. Clearly, further studies of the chaperone properties of SAP and how they might relate to known ECs will be valuable. Of particular interest in the current context, SAP has been found present in all amyloid deposits examined (Breathnach *et al.,* 1981; Coria *et al.,* 1988; Kalaria *et al.,* 1991; Yang *et al.,* 1992). The interaction between SAP and amyloid fibrils is highly specific, and the abundance of SAP in amyloid fibrils relative to its trace concentration in plasma is extraordinary (Botto *et al.,* 1997). SAP has a protease-resistant β-pleated sheet structure that in the presence of Ca^{2+} is resistant to proteolysis (Kinoshita *et al.,* 1992). Furthermore, it has been shown that SAP inhibits the degradation of several types of amyloid fibrils by proteases. Tennent *et al*. (1995) suggested that SAP protects amyloid from proteolytic degradation *in vivo* by binding to fibrils and masking fibrillar conformation.

4.0 THE EFFECTS OF EXTRACELLULAR CHAPERONES ON AMYLOID FORMATION AND TOXICITY

4.1 Effects of ECs on amyloid formation *in vitro*

In vivo amyloid formation and deposition is, in terms of its nature, onset, and progression, an extraordinarily complex process that can occur over much of the lifetime of an individual, thus making it difficult to monitor and study over a reasonable timescale. The co-deposition of other components, including proteins (e.g. molecular chaperones), metals, and glycosaminoglycans, adds further complexity to

the situation. Such complexity prevents the amyloid-forming process from being fully reproduced in a test tube. Nevertheless, by examining indvidual steps in the process *in vitro*, it is still possible to gain insight into many facets of *in vivo* amyloid formation and deposition. *In vitro,* conditions can be manipulated such that fibril formation can be assessed in a manageable timeframe. Perturbation of the environment, with the specific aim of increasing the population of aggregation-prone intermediates, is a common strategy that usually involves, but is not limited to, the use of polypeptide concentrations that exceed physiological levels, alterations to pH or ionic strength, the use of denaturants, or incubation at high temperatures. Much of the current knowledge regarding the effects of ECs on amyloid formation has been gleaned from *in vitro* studies that utilise such strategies.

The *in vitro* chaperone actions of clusterin, haptoglobin, α2M, and SAP, have been previously reported (see section 3.0). Whilst many studies have highlighted the ability of these EC to suppress amorphous aggregation, their effects on amyloid formation are, apart from clusterin, less well-documented. A report published in 1994 provided the first indication that clusterin is capable of preventing amyloid fibril formation (Oda *et al.,* 1994). In this report, it was shown that the formation of Aβ fibrils could be inhibited by sub-stoichiometric levels of clusterin, a finding which has since been confirmed by other independent studies (Matsubara *et al.,* 1996; Hughes *et al.,* 1998). Since then, clusterin has been shown to potently inhibit, in a dose-dependent manner, the formation of fibrils derived from a broad range of other unrelated 'substrates', including prion protein $Pr_{106-126}$ (McHattie and Edington 1999), Apo C-II (Hatters *et al.,* 2002), chicken egg white lysozyme, calcitonin, κ-casein, α-synuclein, β_2-microglobulin, PI3-SH3, Aβ, a model peptide $CC\beta_w$ (Yerbury *et al.,* 2007), and the naturally occurring amyloidogenic variant of lysozyme, I56T (Kumita *et al.,* 2007). Similar inhibitory capabilities have also been documented for α2M. Like clusterin, sub-stoichiometric levels of α_2M (at a 1:8 molar ratio of α2M:substrate) were enough to completely inhibit the formation of fibrillar Aβ aggregates (Hughes *et al.,* 1998). Finally, it was shown that *in vitro* a 1:5 molar ratio of SAP to target protein completely attenuated the fibrillation of $A\beta_{1-42}$ and α_1-antitrypsin-derived C-terminal peptides (Janciauskiene *et al.,* 1995). There are no published data to show whether haptoglobin has similar anti-amyloidogenic properties *in vitro*.

The ability of sub-stoichiometric levels of clusterin, α_2M, or SAP to inhibit fibrillogenesis suggests that the substrate species involved is not highly populated but is crucial for fibril formation. Studies probing the mechanisms of clusterin's chaperone action have provided some indications as to the identity of such species. Recent studies exploring the interactions between clusterin and amyloid forming proteins revealed that clusterin did not bind to the native form of the substrates tested, nor did it interact with mature fibrils (Kumita *et al.,* 2007; Yerbury *et al.,* 2007). Interestingly, the greatest amount of anti-amyloidogenic effect afforded by clusterin was detected when it was present during the early stages of fibrillogenesis. Yet, based on results obtained by mass spectrometry, the presence of clusterin did not eliminate the appearance of a partially unfolded monomeric intermediate identified as the initial step in the pathway of lysozyme amyloidosis (Kumita *et al.,* 2007). The addition of clusterin during the elongation phase did not greatly alter the kinetics of aggregation of I56T lysozyme (Kumita *et al.,* 2007). Similarly, clusterin was more effective at suppressing fibril formation in solutions of Aβ and SH3 which had been seeded with

samples taken from corresponding aggregation mixtures during the lag phase compared with those taken from the late (plateau) phase (Yerbury *et al.,* 2007). This is consistent with data showing that at the same molar clusterin:substrate ratio, as the concentration of amyloid forming protein is increased, clusterin is less able to inhibit the formation of amyloid by Apo C-II, PI3-SH3 and Aβ. The increased concentration of amyloid forming protein increases the abundance of destabilised monomer, favouring self-association into oligomers able to nucleate the aggregation process and thereby shortening the lag phase (Hatters *et al.,* 2002; Yerbury *et al.,* 2007).

Taken together, the above results suggest that clusterin primarily exerts its effects on amyloid formation at or near the nucleation stage. It is likely to be a destabilized pre-fibrillar oligomeric species that are targets for binding by clusterin. During amyloid formation, conformationally rearranged proteins and oligomeric species are usually present at concentrations low enough to account for the potent sub-stoichiometric effects of the ECs; when in equilibrium with the native fold, destabilised and non-native structures can initiate aggregation when present at an abundance as low as 1% (Canet *et al.,* 2002; Marcon *et al.,* 2005). The binding of ECs such as clusterin and α_2M probably reduces the availability of these species to participate in the nucleation events that normally precedes fibril formation (Fig. (2)).

The broad-range specificity of clusterin for amyloid forming substrates is not only impressive but suggests that the effects of clusterin on amyloid formation may be generic. This suggests that there may be a structural feature common amongst amyloid-forming peptides and proteins that are specifically recognised by the chaperone. Whether this is also true for other ECs is not known. Nevertheless, under experimental conditions that favour the formation of amorphous aggregates, both clusterin and α_2M have been shown to form high molecular weight complexes with denatured proteins, at least in part, through hydrophobic interactions (section 3.1 and French *et al.,* (2007)).

Given that hydrophobicity is considered to be an important universal determinant in the formation of fibrillar protein aggregates (Chiti *et al.,* 2002), it is therefore feasible that the inhibitory effects of clusterin, α_2M, and SAP on *in vitro* amyloid formation can be attributed to similar hydrophobic interactions between the chaperones and their amyloidogenic substrates. In support of this notion, clusterin and α_2M (activated and non activated) seem to preferentially bind intermediate protein species formed during amyloid-related aggregation but do not bind to native structures of the same proteins (Narita *et al.,* 1997; Lauer *et al.,* 2001; Hatters *et al.,* 2002; Mettenburg *et al.,* 2002; Kumita *et al.,* 2007; Yerbury *et al.,* 2007). Five times more clusterin-Aβ complex was formed when clusterin was incubated for 18 h at 37^{o}C with Aβ monomer than was the case when it was incubated under the same conditions with pre-aggregated Aβ (Matsubara *et al.,* 1995). Similarly, α_2M did not initially form complexes with Aβ but under conditions that promote amyloid formation stable α_2M-Aβ complexes were formed after 2 hours (Narita *et al.,* 1997). This pattern of binding may be explained by the different levels of hydrophobicity exposed on the various protein conformations produced at different stages of the amyloid-forming pathway. For example, the level of exposed hydrophobicity on a native protein is generally low with hydrophobic residues buried inside the molecule. However, after the protein has been destabilized some of the originally buried hydrophobic residues will become

accessible to the solvent and perhaps chaperones. Subsequent protein aggregation will again bury most of the hydrophobic residues inside the aggregating structure.

The relationship between α_2M and Aβ is complex due to its two functional conformations. There is no doubt that α_2M binds to Aβ with high affinity. However, there are conflicting reports of the particular α_2M conformations that bind to Aβ. There are several reports claiming that Aβ binds to active α_2M but *not* to native α_2M (Narita *et al.,* 1997; Lauer *et al.,* 2001; Mettenburg *et al.,* 2002). In contrast, others claim that native α_2M binds to Aβ (Du *et al.,* 1997; Hughes *et al.,* 1998). Moreover, native α_2M can inhibit fibril formation (Du *et al.,* 1997; Hughes *et al.,* 1998), providing additional evidence that α2M in its native state can bind Aβ. The binding of Aβ occurs in a site that is distinct from the protease and growth factor binding sites (Mettenburg *et al.,* 2002). It is most probable that the binding occurs to a linear sequence of amino acids, as both activated and native α_2M bind Aβ after they have been denatured (Mettenburg *et al.,* 2002). In a curious combination of functions, activated α_2M seems to be able to feed Aβ inside its "cage" to facilitate its proteolysis by a trapped protease (Qiu *et al.,* 1996; Lauer *et al.,* 2001).

Despite many reports of the ability of ECs to inhibit fibril formation *in vitro*, there are indications to suggest that they may, at least under certain conditions, actually promote the formation or maintain the persistence of amyloid fibrils. α_2M binds to and protects prion protein from degradation by proteinase K; this protection was not exerted by α_2M binding to and inhibiting proteinase K (Adler and Kryukov 2007). In addition, another study showed that α_2M prevents the trypsin-mediated degradation of fibrils composed of either immunoglobulin lambda (λ) light chains or β2M (Gouin-Charnet *et al.,* 1997). Similarly, the calcium-dependent binding of SAP to amyloid fibrils formed from Aβ, serum amyloid A protein or immunoglobulin light chain, protected the fibrils from subsequent protease-mediated degradation (Tennent *et al.,* 1995). This in turn led to suggestions that SAP may promote the persistence of amyloid deposits *in vivo.*

For some protein substrates, at low clusterin:substrate ratios (1:50-1:500), clusterin promotes the formation of aggregates with an increased level of thioflavin T fluorescence, suggesting that (at these ratios) it promoted the formation of fibrils from calcitonin, α-synuclein and Aβ. At these low levels, clusterin was found incorporated into insoluble Aβ and SH3 fibrils (Yerbury *et al.,* 2007). Similarly, at low SAP:Aβ ratios, SAP promoted the formation of Aβ aggregates (Hamazaki 1995); at SAP:Aβ of 1:1000, short fibrillar-like structures lacking typical amyloid features were formed which often contained associated SAP molecules (Janciauskiene *et al.,* 1995). In addition, α_2M has been shown to stabilize a conformation of the prion protein resistant to proteinase K (PrPRes) that is thought to initiate aggregation and is associated with prion disease pathology (Adler and Kryukov 2007). Taken together, these results suggest that when ECs are present at low levels relative to the substrate they may act to stabilize an otherwise unstable conformation and facilitate amyloid fibril formation (Fig. (3)).

4.2 The *in vitro* effects of ECs on amyloid toxicity

Recent studies have indicated that the location of amyloid deposits *in vivo* do not correlate well with sites of neurotoxicity (e.g. in AD (Kirkitadze *et al.,* 2002), ALS

(Lee *et al.,* 2002), Parkinson's disease (Volles and Lansbury 2003) and familial amyloidotic polyneuropathy (Sousa *et al.,* 2001)). Neuronal losses associated with these disorders may be brought about by toxicity exerted by smaller soluble aggregates (sometimes referred to as oligomers or protofibrils; see section 2.2) (Chiti and Dobson 2006). These protofibrils have been shown to be more toxic than both the protein/peptide from which they are made and mature fibrils constructed from them (Bucciantini *et al.,* 2002). If this scenario is correct, then it follows that at least under some conditions EC-mediated inhibition of protein aggregation in the brain could actually promote neurotoxicity. When tested against neuronal cell lines, under certain specific conditions, clusterin and α_2M were shown to promote the neurotoxicity of Aβ (clusterin - PC12 cells (Oda *et al.,* 1995); α_2M - LAN5 cells (Fabrizi *et al.,* 2001)). In stark contrast, using primary rat mixed neuronal cultures, others have demonstrated that clusterin and α_2M can protect cells from Aβ toxicity (Boggs *et al.,* 1996; Du *et al.,* 1997). Even within individual studies, depending on the conditions used, clusterin was shown both to protect cells and to promote cytotoxicity. Complexes of clusterin and Aβ formed at high ratios of clusterin:Aβ (e.g. 1:10) were less toxic to SH-SY5Y cells than Aβ alone; in contrast, complexes formed at low ratios of clusterin:Aβ (e.g. 1:500) were more toxic than Aβ alone (Yerbury *et al.,* 2007). Thus, the effects of ECs, and in particular clusterin, on the toxicity of aggregates are complex and have been shown to depend upon the clusterin:substrate ratio, the stage of amyloid formation at which the aggregates are formed, and the cell type.

It has been postulated that the level of hydrophobicity exposed by aggregates may determine their toxicity (Chiti and Dobson 2006). How this directly relates to the mechanism of their toxicity is uncertain and is currently a hotly researched topic, but it is probably due to interactions of hydrophobic residues with membranes or other molecules essential for normal cellular function (Chiti and Dobson 2006). This, in part, may explain the differential effects of clusterin on toxicity. When the substrate protein is present at a high molar excess, clusterin may be unable to mask all regions of exposed hydrophobicity but instead may stabilize aggregates which retain sufficient exposed hydrophobicity to exert toxicity (Fig. (3)). In contrast, at higher but still sub-stoichiometric ratios of clusterin:Aβ, toxicity is reduced (Yerbury *et al.,* 2007).

Another potentially important factor to consider when trying to understand the *in vivo* effects of ECs on amyloid toxicity, is that in addition to their effects on the nature and size of protein aggregates, ECs may also be involved in the physical clearance of protein aggregates. This is illustrated by the demonstration that in the presence of α_2M (but not otherwise), SH-SY5Y cells expressing the α_2M receptor (LRP) are more resistant to Aβ toxicity than cells that do not (Fabrizi *et al.,* 2001). The protective effect of α_2M could be inhibited by RAP (a pan-specific inhibitor of LRP ligand binding). Furthermore, α_2M promoted Aβ toxicity against LRP-negative LAN5 cells but had the opposite effect with LRP-expressing LAN5 transfectants (Fabrizi *et al.,* 2001). Therefore, although ECs may provide cells with some protection by binding to exposed hydrophobic regions on protein aggregates, a further protective mechanism may only come into play when appropriate cell surface receptors are available to mediate uptake and degradation of EC-substrate protein complexes.

4.3 ECs and protein disposal in the brain

It has been proposed that ECs are scavengers for hydrophobic proteins in the extracellular space, inhibiting protein aggregation and deposition and guiding misfolded proteins to specific receptors for their internalization and subsequent lysosomal degradation ((Yerbury *et al.,* 2005) and see Fig. (3)). This system of EC directed receptor-mediated endocytosis may play a major role in the maintenance of protein solubility in the extracellular fluids in the brain (interstitial fluid and CSF). Evidence supporting this model includes:
(1) In the CNS, ECs are produced locally in astrocytes and some neuron populations (Lauro *et al.,* 1992; Zwain *et al.,* 1994; Klimaschewski *et al.,* 2001) and can be transported from plasma across the blood brain barrier (BBB) (Zlokovic 1996). The receptors LRP and megalin are expressed on the surface of cells in contact with CSF (i.e. chloroid plexus and ependymal cells (Kounnas *et al.,* 1994) and at the BBB (Zlokovic 1996; Donahue *et al.,* 2006) and LRP is also found on the surface of astrocytes (Arelin *et al.,* 2002) and microglial cells (Laporte *et al.,* 2004).
(2) Clusterin and α2M are both found complexed with soluble amyloid-forming proteins in human CSF (Ghiso *et al.,* 1993; Adler and Kryukov 2007).
(3) It has previously been shown that clusterin-Aβ complexes bind to megalin on the surface of mouse teratocarcinoma F9 cells, and are subsequently internalised, transported to lysosomes and degraded (Hammad *et al.,* 1997). Similarly, α2M-Aβ complexes are internalized via LRP expressed on U87 cells and are subsequently degraded (Narita *et al.,* 1997).
(4) The normally rapid removal of radiolabelled Aβ from mouse brain is significantly inhibited by the LDL family inhibitor RAP and antibodies against LRP-1 and α2M (Shibata *et al.,* 2000). Furthermore, when complexed with clusterin, the rate of clearance of $A\beta_{1-42}$ from the mouse brain across the BBB into plasma is increased by more than 80% and this transport is significantly inhibited by anti-megalin antibodies (Bell *et al.,* 2007).

4.4 When quality control is compromised?
If a system of extracellular protein quality control (QC) operates and is efficient, why do protein deposits sometimes develop? Protein aggregation in the brain is generally age-related, and some proteins such as Aβ accumulate as a part of normal aging (Fukumoto *et al.,* 1996). In young, healthy humans the disposal of proteins such as transthyretin or peptides like Aβ is efficient (that is there are no apparent protein deposits). Thus, it is likely that protein deposition may represent an age-dependent overloading of the QC system that ultimately results in toxicity and neurodegeneration. If the QC system is overloaded in amyloid diseases of the brain, it is critical to pinpoint the factors and pathways involved to facilitate identification of new potential therapeutic strategies.

One poorly understood pathway relates to the observation that under some conditions ECs can actually promote the formation of amyloid fibrils; clusterin, α2M and SAP have been shown to promote fibril formation *in vitro* (Hamazaki 1995; Adler and Kryukov 2007; Yerbury *et al.,* 2007) (see section 4.1). In addition, in a transgenic mouse model expressing APP (PDAPP mice), a comparison of clusterin -/- and clusterin +/+ mice showed no difference in the amount of Aβ deposited in the brain, however the amount of thioflavin S positive material was larger in clusterin +/+ mice (DeMattos *et al.,* 2002). Although there was no electron microscopy data to confirm that this was in fact amyloid fibrils, the authors suggest that this demonstrates

clusterin promoted fibril formation in this model. In contrast, PDAPP *apoE−/−*, *clusterin−/−* mice had significantly increased Aβ levels in CSF and intersititial fluid and thioflavin–S deposits in the brain (DeMattos *et al.,* 2004). The results of this latter study were interpreted as suggesting that clusterin works cooperatively with ApoE to inhibit the formation of Aβ amyloid in the brain. The interpretations presented from these two studies are seemingly contradictory, and a clear understanding of the *in vivo* role of clusterin in amyloidogenesis has yet to be achieved. An important factor influencing the nature of the effect of clusterin on amyloid formation, at least *in vitro*, is the clusterin:substrate protein ratio. At very low ratios of clusterin:substrate, clusterin has been shown to enhance amyloid associated thioflavin T fluorescence suggesting that it increases amyloid formation, while at higher but still sub-stoichiometric ratios have the opposite effect (see section 4.1). A similar biphasic effect on amyloid formation has also been shown for the intracellular yeast chaperone hsp104 (Shorter and Lindquist 2004).

It is possible that at times in local microenvironments of the CNS, the concentrations of ECs are low and that under these conditions they actually promote the formation of amyloid fibrils. Interestingly, under conditions of high substrate protein excess, clusterin becomes incorporated into insoluble aggregates (Yerbury *et al.,* 2007). Sequestration of clusterin into deposits would effectively lower it's availability, and potentially further promote fibril formation. If the other ECs behave similarly, then it is no surprise that all are found associated with plaques (see Table (1), and Fig. (3)). In humans with AD (Duguid *et al.,* 1989; May *et al.,* 1989) and Creutzfeldt Jacob disease (Duguid *et al.,* 1989; Sasaki *et al.,* 2002) the levels of clusterin mRNA are increased, however, there is no associated increase in clusterin protein levels in AD CSF (Lidstrom *et al.,* 2001) and the level of clusterin protein in CSF is decreased by 2.5 fold in Creutzfeldt Jacob disease (Piubelli *et al.,* 2006). In addition, decreased levels of clusterin in the eye are thought to be responsible for deposition of Pseudo exfoliation (PEX) material in PEX syndrome (Zenkel *et al.,* 2006) (again clusterin is found associated with PEX deposits).

Another factor that could favour the development of amyloid pathology is a dysfunction in the expression or function of relevant cell surface receptors (see Fig. (3)). Under some conditions, ECs may stabilize toxic conformations of substrate proteins with exposed hydrophobicity (see section 4.2). If receptor-mediated clearance is inadequate, the persistence of such toxic complexes could manifest as neuronal damage. *In vitro* assays demonstrated that when neuronal cells were in mixed cultures with astrocytes and microglia (which express receptors capable of specifically binding to ECs, e.g. LDLR family members), by complexing with Aβ, both clusterin and α2M were neuroprotective. However, when neuronal cells were cultured alone, complexes of clusterin or α2M with Aβ were neurotoxic, suggesting that complexes of EC and aggregating protein may be toxic in the absence of receptor mediated endocytosis pathways. Moreover, there are increased plasma concentrations of several LRP ligands in AD patients, including ApoE, α1 anti-chymotrypsin and urokinase (Aoyagi *et al.,* 1992; Licastro *et al.,* 1995; Taddei *et al.,* 1997) suggesting that LRP may be either overwhelmed, downregulated or faulty in AD. More recently, this has been confirmed by data showing that LRP expression at the BBB is decreased in AD patients and mouse models (Deane *et al.,* 2004). As discussed above (section 4.3), this is consistent with the results from mouse models where antibody-mediated

blockade of megalin and LRP significantly prolonged the time taken to clear Aβ from the brain (Shibata *et al.,* 2000; Bell *et al.,* 2007).

5.0 CONCLUSION & FUTURE DIRECTIONS

In recent years there has been considerable progress in our understanding of protein deposition disorders. In particular our understanding of the nature and significance of amyloid formation and deposition and the role that these play in disease has taken dramatic leaps forward. Further advances in understanding of the mechanisms which control extracellular protein folding are likely to identify new strategies for effective disease therapies. This chapter focused on what little is known about quality control of the folding of amyloid forming proteins in the extracellular space of the brain. Since the EC field is still in its infancy, this chapter is necessarily speculative in a number of areas. However, it is quite clear that amyloid forming proteins such as Aβ and prion proteins are 'chaperoned' in the brain by what have become termed extracellular chaperones. It is proposed that once bound to misfolded proteins, extracellular chaperones guide them to specific receptors that function to direct proteins into lysosomes for degradation. In this scenario, specific receptors such as LRP and megalin are an integral and critical part of a quality control system which provides protection against dangerously hydrophobic proteins/peptides in the brain. Since relatively little is known about ECs and their role in protein deposition diseases many future directions are possible. Initially, it will be important to better understand the mechanism by which, under some conditions, ECs promote amyloid formation, as this has clear relevance to the development of any EC-based therapies. Certainly, it will be critical to evaluate the roles of ECs in a variety of *in vivo* models of protein deposition diseases. It may be possible to accelerate the pace of advances by utilizing innovative new disease models such as those developed using the fruit fly *Drosophila*. The short generation times and ease of genetic manipulation in these models create opportunities not accessible with mammalian systems. Much remains to be discovered, ensuring that exciting times lie ahead.

Table 1. Summary of protein deposition disorders in the CNS and the extracellular chaperones known to be associated with them.

Disease	Aggregating protein/peptide	Location	EC associated with deposit
Alzheimer's disease	Aβ peptide	Extracellular	Clus (Calero *et al.,* 2000), α_2M (Fabrizi *et al.,* 2001), Hp (Powers *et al.,* 1981), SAP (Perlmutter *et al.,* 1995)
Spongiform encephalopathies	Prion Protein	Extracellular	Clus (Chiesa *et al.,* 1996; Freixes *et al.,* 2004), α_2M (Adler and Kryukov 2007), SAP (Ishii *et al.,* 1984)
Parkinson's disease	α-synuclein	Intracellular	Clus (Sasaki *et al.,* 2002), SAP (Kalaria and Grahovac 1990), α_2M(Nicoletti *et al.,* 2002)
Dementia with lewy bodies	α-synuclein	Intracellular	Clus (Sasaki *et al.,* 2002), SAP (Kalaria and Grahovac 1990)
Frontotemporal dementia with Parkinsonsism	Tau	Intracellular	Unknown
Amytrophic lateral sclerosis	Superoxide dismutase 1	Intracellular	Unknown
Huntington's disease	Huntingtin with poly Q expansion	Intracellular	Unknown
Spinocerebellar ataxia's	Ataxins with poly Q expansion	Intracellular	Unknown
	TATA box-binding protein with poly Q expansion	Intracellular	Unknown
Spinal and bulbar muscular atrophy	Androgen receptor with poly Q expansion	Intracellular	Unknown
Hereditary dentatorubral-pallidoluysian atrophy	Atrophin-1 with poly Q expansion	Intracellular	Unknown
Familial British dementia	ABri	Extracellular	Clus (Ghiso *et al.,* 1995), SAP (Rostagno *et al.,* 2007)
Familial Danish Dementia	ADan	Extracellular	Clus (Lashley *et al.,* 2006), SAP (Rostagno *et al.,* 2007)
Familial amyloid polyneuropathy	TTR	Extracellular	Unknown
Age related macular degeneration	Drusen components	Extracellular	Clus (Sakaguchi *et al.,* 2002), SAP (Ambati *et al.,* 2003)
Gelatinous drop-like corneal dystrophy	Keratoepithelin	Extracellular	Clus (Nishida *et al.,* 1999), SAP (Stix *et al.,* 2005)
Lattice type I corneal dystrophy	M1S1	Extracellular	Clus (Nishida *et al.,* 1999), SAP (Stix *et al.,* 2005)
Pseudoexfoliation (PEX) syndrome	PEX components	Extracellular	Clus (Zenkel *et al.,* 2006), SAP (Schlotzer-Schrehardt *et al.,* 1992)
Down's syndrome	Aβ	Extracellular	Clus (Kida *et al.,* 1995), SAP (Kalaria and Grahovac 1990)

Figure 1. A schematic representation of some of the fates that await polypeptide chains *in vivo*. Newly synthesized polypeptides fold into their biologically active, native conformation, often via the transient formation of one or more partially unfolded intermediates. Failure to do so may result in the polypeptide experiencing other fates, including degradation or irreversible aggregation to form either disordered (amorphous) aggregates or highly organized amyloid fibrils. The formation of amyloid fibrils occurs via a nucleation dependent mechanism in which soluble prefibrillar oligomers or nuclei are formed during the lag phase. These oligomeric species then act as templates to sequester other aggregation-prone intermediates, leading to rapid fibril growth (represented by the exponential elongation phase) and subsequently, to the formation of insoluble mature amyloid fibrils. The plateau phase represents the steady state when maximum fibril growth has been reached. Adapted from (Chiti and Dobson 2006).

Figure 2. Possible consequences for proteins when extracellular quality control mechanisms fail. An extracellular quality control process has recently been proposed whereby extracellular chaperones (EC) facilitate the clearance of non-native protein structures via receptor-mediated endocytosis and intracellular, lysosomal degradation. The existence of such a quality control mechanism ensures that proteins do not persist beyond their intended lifespan in the extracellular space. Failure of this system, at any of its stages, may potentially result in the onset of disease caused by the accumulation and/or deposition of i) cytotoxic species generated through the amyloid assembly pathway, ii) aberrantly folded structures and aggregates such as insoluble complexes of ECs and fibrillar material, or iii) degradation-resistant complexes (as exemplified by $A\beta_{1\text{-}42}$ amyloid fibrils).

Figure 3. The biphasic effects of chaperones on amyloid fibril formation. Under certain conditions, proteins readily self-associate into highly structured amyloid fibrils. The kinetics of the amyloid assembly process is often depicted as a sigmoidal curve to reflect the three distinct phases (i.e. lag, elongation and plateau; see also Figure 1) that characterizes the fibril formation pathway. This kinetic profile can be detected *in vitro* using real-time spectroscopic techniques, such as those that monitor fibril formation by measuring the relative binding of amyloid-specific dyes such as Congo-red and Thioflavin T. Such techniques have, in the past, provided clues as to what effects chaperones have on fibril formation. Using various model substrate proteins, it was shown that complete inhibition of fibril formation can be achieved when clusterin and α2M are present at high (but usually still sub-stoichiometric) levels relative to the substrate protein. Yet, at low EC:substrate ratios, extensive amyloid or amyloid-like fibril formation is observed for some substrate proteins, suggesting that in these cases, ECs may be stabilizing an otherwise unstable conformation and promoting amyloid fibril formation

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FIGURE 1

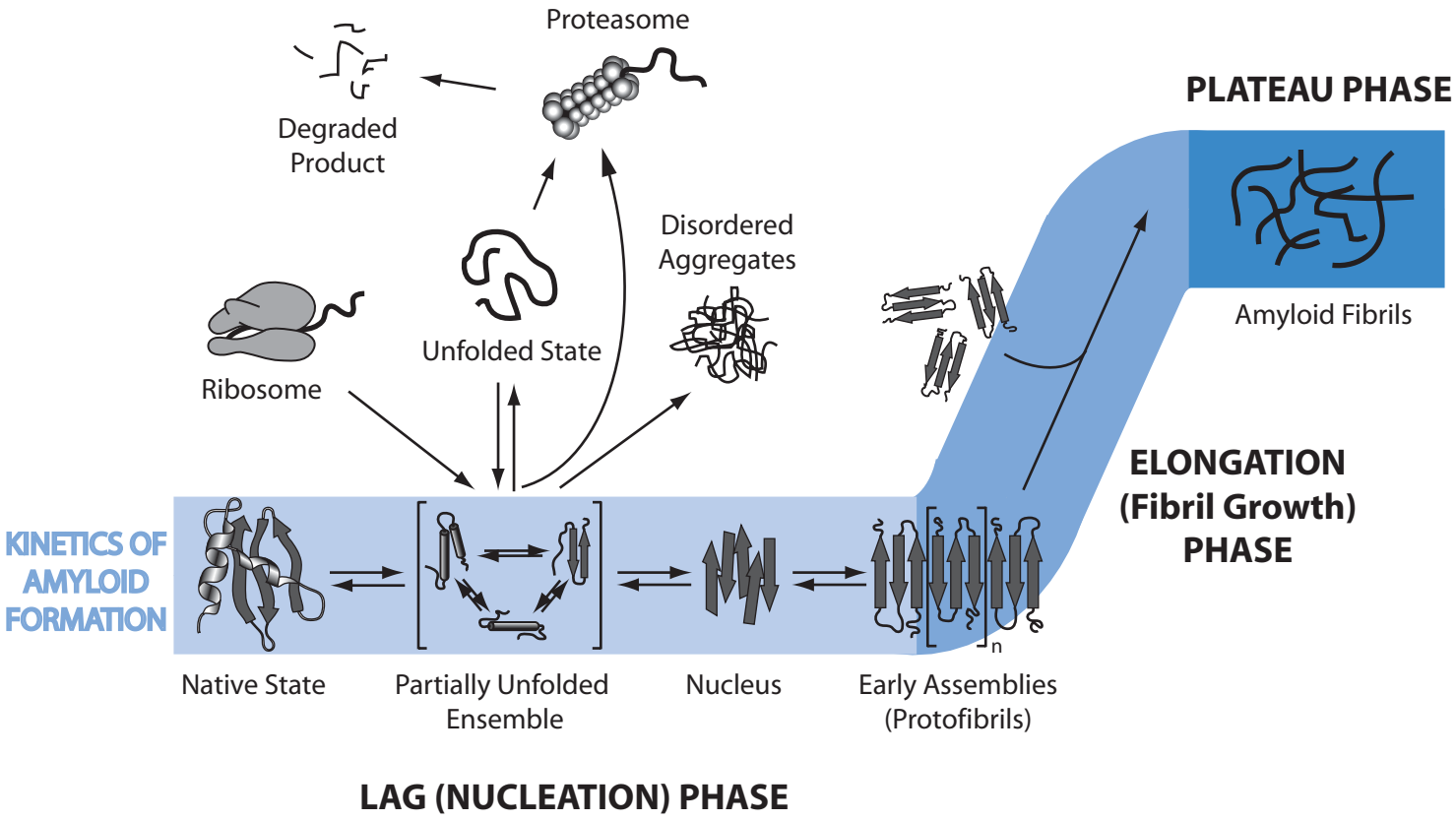

Proteasome
Degraded Product
PLATEAU PHASE
Disordered Aggregates
Amyloid Fibrils
Unfolded State
Ribosome
ELONGATION (Fibril Growth) PHASE
KINETICS OF AMYLOID FORMATION
n
Native State
Partially Unfolded Ensemble
Nucleus
Early Assemblies (Protofibrils)
LAG (NUCLEATION) PHASE

FIGURE 2

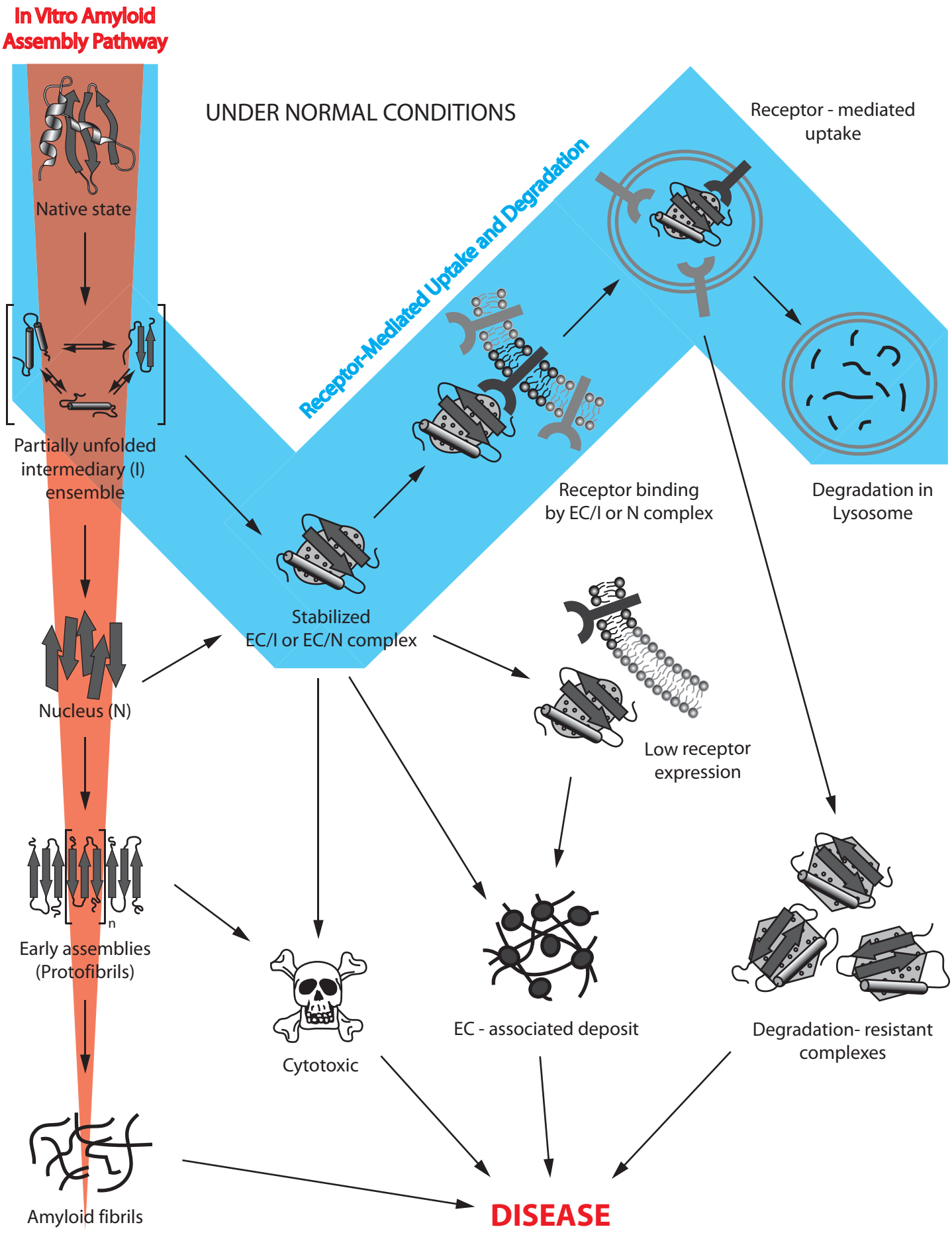
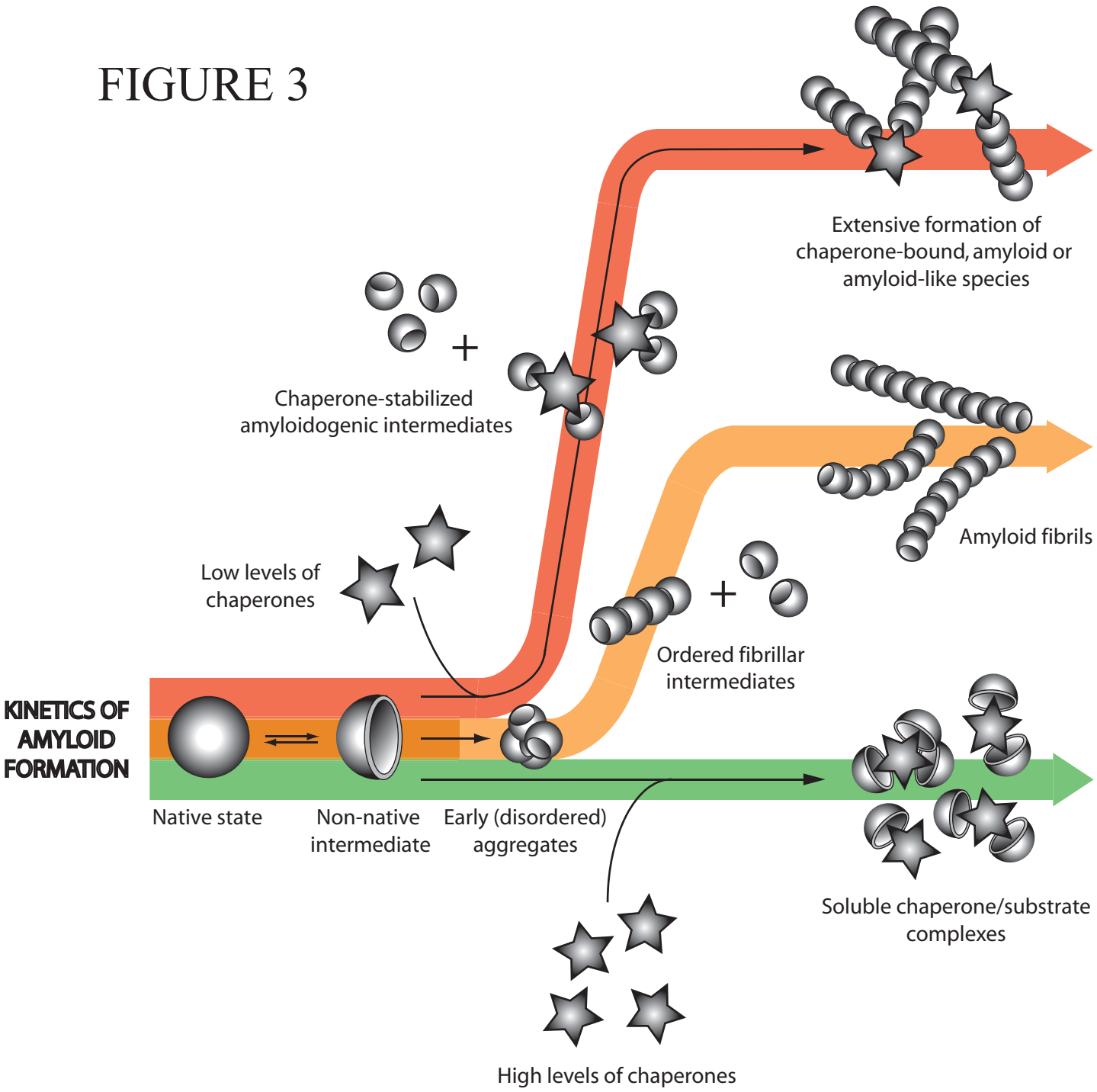

In Vitro Amyloid Assembly Pathway
UNDER NORMAL CONDITIONS
Native state
Partially unfolded intermediary (I) ensemble
Receptor-Mediated Uptake and Degradation
Receptor - mediated uptake
Receptor binding by EC/I or N complex
Degradation in Lysosome
Stabilized EC/I or EC/N complex
Nucleus (N)
Low receptor expression
Early assemblies (Protofibrils)
Cytotoxic
EC - associated deposit
Degradation- resistant complexes
Amyloid fibrils
DISEASE
WHEN QUALITY CONTROL IS COMPROMISED

FIGURE 3


Extensive formation of chaperone-bound, amyloid or amyloid-like species
Chaperone-stabilized amyloidogenic intermediates
Amyloid fibrils
Low levels of chaperones
Ordered fibrillar intermediates
KINETICS OF AMYLOID FORMATION
Native state
Non-native intermediate
Early (disordered) aggregates
Soluble chaperone/substrate complexes
High levels of chaperones